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Respiration of Wild Type and Extrachromosomal Mutants of *Neurospora crassa*[1]

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The specific rates of respiration of cells of wild type and four extrachromosomal mutants of *Neurospora crassa* were measured throughout the vegetative growth cycle. Two forms of respiration were observed: (i) cyanide sensitive; and (ii) cyanide resistant, salicyl hydroxamate sensitive. These two forms are called terminal and alternate, respectively. The former proceeds by the mitochondrial electron transfer chain and involves the cytochromes; the latter apparently proceeds by the initial portion of the electron transfer chain and does not involve cytochromes. Large and rapid changes of both the terminal and alternate respiratory activities occurred during the vegetative growth cycle. The kinetics of these changes in wild type were compared under some conditions which inhibit protein synthesis and others in which the nitrogen source was varied. The kinetics of the changes of the two forms of respiration of mutants differed from those normally exhibited by wild type, but with varied experimental conditions wild type could be made to resemble the mutants. The results of these studies are discussed in terms of a dynamic model of regulation of mitochondrial biogenesis in the coordination of the synthesis of mitochondrial proteins encoded by nuclear and mitochondrial genomes.

Cellular respiration of higher plants (3) and of some simple eukaryotes such as fungi (10, 13-18, 26, 27; B. L. Sauer, H. J. Colvin, and K. D. Munkres, Biochem. Biophys. Res. Commun., in press) may proceed by two pathways. The relationship of these pathways in mitochondria is shown in Fig. 1. Electrons may be transferred to oxygen by either the mitochondrial cytochrome chain (terminal respiration) or by an alternate oxidase. The latter carries electrons from the proximal portion of the electron transfer chain to oxygen prior to cytochrome *b* and is resistant to inhibition by either cyanide or antimycin, but sensitive to hydroxamates.

The biochemical and physiological consequences of slow-growing extrachromosomal mutations in *Neurospora crassa* have been investigated in several laboratories. Tissières et al. (29) noted that old mycelia of the mutant [*mi-1*] (poky) respired at a rate one-third to one-half that of wild type. Unlike wild type, most of the mutants respiration was resistant to both cyanide and azide. Subsequently, it was demonstrated that mitochondria isolated from young mycelia of this mutant, unlike those of wild type, were resistant to azide in the oxidation of succinate (8). Lambowitz and co-workers (16, 17) observed that the specific respiratory rate of mutant mycelia actually exceeded that of wild type in early phases of growth. This observation was confirmed and extended by Howell and Munkres (manuscript in preparation). Conidia of 14 mutants, including [*mi-1*], exhibited specific respiratory rates that were 6- to 18-fold greater than that of wild type. (The mutants were relatively slow growing and yielded colonies which were deficient in the capacity to reduce triphenyltetrazolium to formazan.) The respiration of the mutants, unlike that of wild type, was resistant to cyanide and antimycin (16; B. L. Sauer et al., in press; N. H. Howell and K. D. Munkres, in preparation) and sensitive to salicyl hydroxamate (16; B. L. Sauer et al., in press). However, when wild-type cells of either *N. sitophila* (26) or *N. crassa* (16) were incubated with the respiratory inhibitor antimycin A, they adapted and respired by a pathway that was resistant to antimycin A. This adaptive respiration of wild type, like the constitutive antimycin A-resistant respiration of the mutants, was also resistant to cyanide (16; N. H. Howell and K. D. Munkres, in

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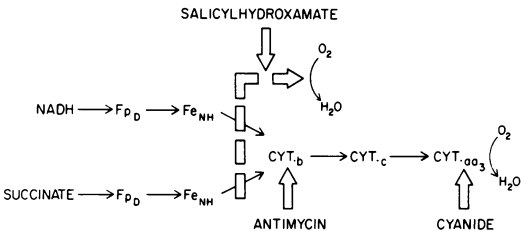

FIG. 1. *Pathways of mitochondrial respiration in fungi and higher plants. Solid arrows indicate sites of inhibitor action. The broken arrow indicates the terminal step of the alternate oxidase.* F_{pD}, *flavoprotein dehydrogenase.* Fe_{NH}, *non-heme iron protein.*

preparation) and sensitive to salicyl hydroxamate (16). Similarly, incubation of wild-type cells with cyanide led to the appearance of cyanide-resistant respiration (16; N. H. Howell and K. D. Munkres, in preparation). In addition, incubation of wild-type mycelia with chloramphenicol, an inhibitor of mitochondrial protein synthesis, led to an increase in the activity of the alternate oxidase (16). Thus, it appeared that wild-type respiration normally proceeds by the mitochondrial cytochrome chain, but that an alternate oxidase diverging from that chain prior to cytochrome *b* (17) is inducible by genetic or chemical perturbations. The respiration of the mutants appeared to proceed primarily by way of an alternate oxidase which was apparently constitutive.

The observation of the inducible, alternate oxidase in wild type, the observations of changes in activities of mitochondrial electron transfer enzymes and cytochromes during the growth cycle (4), and the observations that the nitrogen source affects the quantity and form of respiration (B. L. Sauer et al., in press) suggested, however, that the form and quantity of respiration may be subject to temporal and nutritional regulation. The results of the present studies support that conclusion. The activities of the two forms of respiration in wild type and four extrachromosomal mutants were found to undergo large changes during the vegetative growth cycle. The programs for these changes in mutants differed from that of wild type. The results are interpreted in terms of a model of dynamic control of regulation of mitochondrial biogenesis.

MATERIALS AND METHODS

Strains. Terms enclosed in brackets denote the cytoplasmic genotype. The following were obtained from R. K. Littlewood: wild-type RL 1256*A* [+] (also called 74*A*-8); RL 1250*a*, *al-2*, f^- [*mi-1*]; RL 3603-1-1*a*, f^+ [*mi-1*]; RL 1385*A*, *ylo-1*, [*mi-3*]; and RL 1200*A*, *arg-5*, [*SG-1*]. All of the cytoplasmic mutants had been back-crossed seven times as protoperithecial parent to 74*A*-8.

Cultures. For conidial production, rarely a minimal medium, Fries (2), and generally YECE (11), were used. YECE is an enriched medium containing yeast extract, Casamino Acids, and salts. Erlenmeyer flasks (125 ml) containing 20 ml of medium supplemented with 2% glucose and 2% agar were inoculated with conidia, incubated at 30 C for 3 to 4 days in dim light, and then incubated at room temperature for 3 to 9 days in continuous light.

Conidia were washed from the flasks with Fries medium containing 2% glucose. The suspension was filtered through glass wool and adjusted to an optical density of 0.30 to 0.40 at 550 nm by dilution with the colorless medium. A 10-ml amount of the conidial suspension was added to each of a series of sterile, silicon-treated 25-ml Erlenmeyer flasks. Details of the silicon treatment of the flasks are described elsewhere (H. J. Colvin and K. D. Munkres, Neurospora Newslett., in press). (This treatment was necessary to prevent mycelia from sticking to the glass with consequent inhomogeneous growth.) The inoculated flasks were incubated at 30 C on a rotary shaker at 270 rpm.

Dry weight measurements. The procedures for the collection of cells and measurement of their dry weight are described elsewhere (H. J. Colvin and K. D. Munkres, in press). By these procedures, the coefficients of variation of dry weights from replicate flasks of wild type were 10% during the first 4 h of culture and 1 to 3% thereafter.

Respiration. Respiratory rates were measured with a polarograph as previously described (B. L. Sauer et al., in press) and expressed as Q_{O_2} (microliters of O_2 per hour per milligram dry weight). The conditions for the inhibition of respiration with cyanide and salicyl hydroxamate are described elsewhere (B. L. Sauer et al., in press).

RESULTS

Growth. The logarithmic growth rates and maximal dry weights of the mutants, except [*SG-1*], were less than those of wild type (Fig. 2, Table 1). No differences in the time of the lag period before the onset of logarithmic growth were observed.

Terminal respiratory activity. The changes in specific terminal (cyanide-sensitive) respiratory activities of wild type and mutants during the vegetative growth cycle are shown in Fig. 3. These activities are normalized relative to growth rates in Fig. 4.

In wild type, the rate of increase of specific terminal respiratory activity was a linear function of the number of mass doublings up to four (Fig. 4). The synthesis of the terminal system then apparently ceased, and the next mass doubling led to about a 50% reduction in specific activity. In stationary phase, the activity declined to zero by 55 h (Fig. 3).

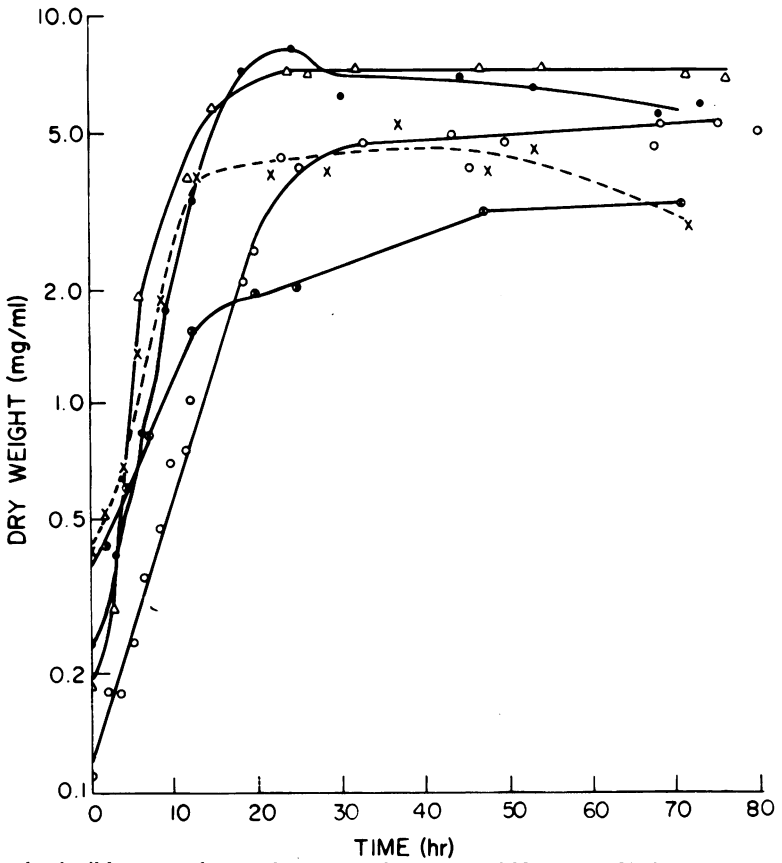

FIG. 2. *Growth of wild type and extrachromosomal mutants of N. crassa. Shake cultures at 30 C in Fries minimal containing initially 2% (wt/vol) glucose. Symbols: Δ, wild type; O, [mi-3]; ⊗, [mi-1]f⁻; ×, [mi-1]f⁺; ●, [SG-1].*

TABLE 1. *Logarithmic growth rates and maximal biomass of wild type and extrachromosomal mutants of N. crassa*[a]

Strain	Mass doubling time (h)	Maximal biomass (mg dry weight/ml, 80-h culture)
Wild-type, 74A8	2.2	7.7
Mutants:		
[*mi-1*]*f*⁻	6.0	2.5
[*mi-1*]*f*⁺	3.5	5.0
[*mi-3*]	3.6	5.5
[*SG-1*]	2.7	7.0

[a] In shake culture of Fries medium with 2% (wt/vol) initial glucose concentration at 30 C. Other conditions were as described in Materials and Methods and by H. J. Colvin and K. D. Munkres (in press).

The initial rates of increase of specific terminal respiratory activities of the mutants approached the rate of wild type, but unlike wild type the increase was not sustained for more than one to two doublings (Fig. 4). Then during the next two to four doublings, the mutant activities declined at rates which were greater than those expected by dilution from the increment in mass.

Two or three cycles of increase and decrease of activity were exhibited by [*mi-1*]*f*⁺, [*mi-3*], and [*SG-1*], but wild type and [*mi-1*]*f*⁻ exhibited only one cycle (Fig. 3).

After 80 h of culture, terminal respiratory activity in both wild type and mutants was not detectable.

In wild type, chloramphenicol completely inhibited the increase in terminal activity with little or no inhibition of growth (Fig. 5A). After the addition of chloramphenicol at either 0 or 2 h, the terminal activity remained constant for 2 to 6 h and then decreased by 20- to 80-fold in 2 h. Since the mass doubling times were 2.5 to 3.5 h, these decreases were greater than expected by dilution from increment in cell mass.

The composition of the preculture medium used for conidial production had a marked

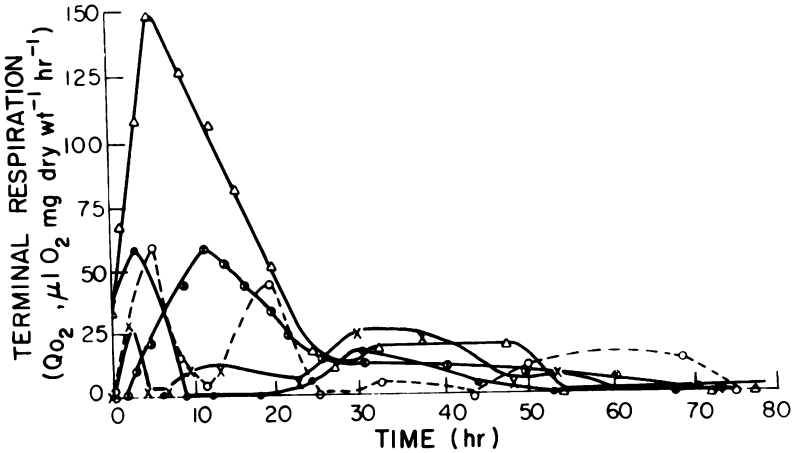

FIG. 3. *Specific terminal respiratory activities of mycelia of wild type and extrachromosomal mutants at various culture ages. Conditions and symbols as in Fig. 2. Terminal respiration is defined as that fraction of the total respiration which is cyanide sensitive (see Materials and Methods).*

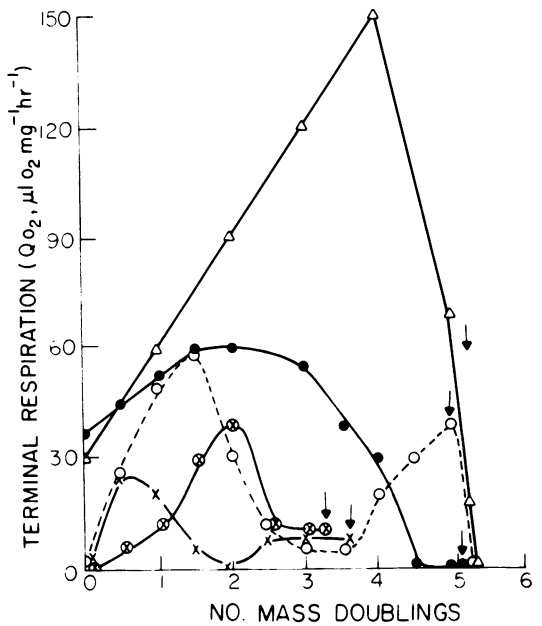

FIG. 4. *Specific terminal respiratory activities of mycelia of wild type and extrachromosomal mutants as a function of number of mass doublings. Conditions and symbols as in Fig. 2 and 3. The arrows indicate the beginning of stationary phase.*

effect upon terminal respiratory activity of wild-type conidia (B. L. Sauer et al., in press) and the rate and magnitude of change of this activity during subsequent germination and growth (Fig. 6). The two preculture media employed here, YECE and Fries, differ primarily in ammonium ion concentration; the former was low and the latter was high (B. L. Sauer et al., in press).

Alternate respiratory activity. The changes in specific alternate (cyanide resistant, salicyl hydroxamate sensitive) respiratory activities of wild type and mutants during the vegetative growth cycle are shown in Fig. 7. These activities are normalized relative to growth rates in Fig. 8.

In wild type, specific alternate respiratory activity was very low until the beginning of stationary phase (17 h), when it increased to a Q_{O_2} of 25 and then remained constant until at least 80 h (Fig. 7 and 8).

All of the mutants exhibited large and rapid changes in alternate respiratory activity. Their maximal activities exceeded that of wild type by at least 100-fold (Fig. 7 and 8). After two to three doublings, the activities of [*mi-1*]f^- and [*mi-3*] approached a maximum. The subsequent doublings before stationary phase then led to a dilution of activity equivalent to the increment in mass. In [*SG-1*], however, the increase in alternate activity persisted until stationary phase; thereafter the activity declined rapidly.

Two or three cycles of increase and decrease of alternate activity were observed in [*mi-1*]f^+, [*mi-3*], and [*SG-1*], but wild type and [*mi-1*]f^- exhibited only one cycle (Fig. 7).

After 80 h of culture, alternate respiratory activities in wild type and mutants approached a similar order of magnitude (Fig. 7).

In wild type, the addition of chloramphenicol led to at least a 160-fold increase in alternate activity within 6 h (Fig. 5B). Since the mass doubling times were 2.5 to 3.5 h, the rate of increase of alternate activity exceeded the growth rate by 40-fold. Therefore, chloramphenicol led to a derepression of the synthesis of

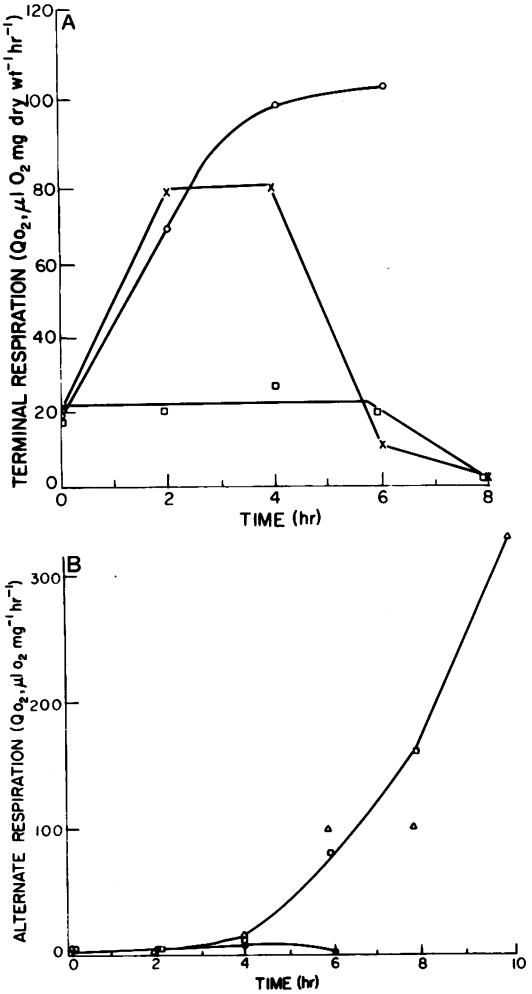

FIG. 5. *(A) Inhibition by chloramphenicol of the increase in terminal respiratory activity of wild-type mycelia. Culture conditions as in Fig. 2. Symbols:* O, *no chloramphenicol;* ×, *chloramphenicol (2 mg/ml) added at 2 h;* □, *chloramphenicol (2 mg/ml) added at beginning of culture. The dry weight doubling times were: 2.5, 3.5, and 3.5 h, respectively. (B) Derepression by chloramphenicol of the increase of alternate respiratory activity of wild-type mycelia. Conditions as in Fig. 5A. Symbols:* O, *no chloramphenicol;* Δ, *chloramphenicol (2 mg/ml) added at 2 h;* □, *chloramphenicol (2 mg/ml) added at beginning of culture.*

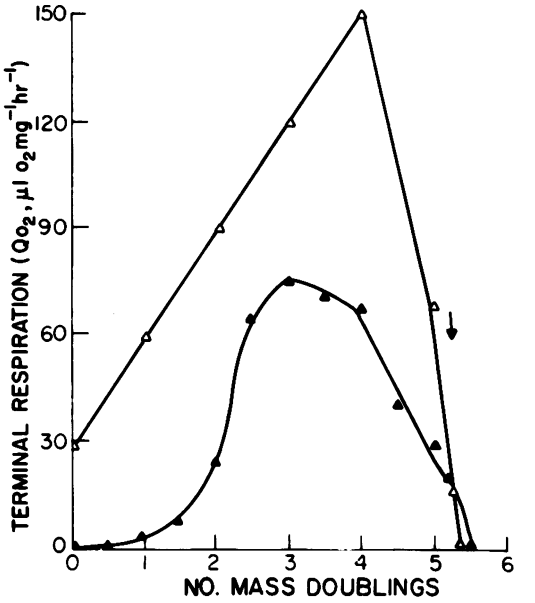

FIG. 6. *Influence of preculture medium upon the change of terminal respiratory activity during growth of wild type. Conidia of wild type were obtained from slants of YEGCE (low ammonia,* Δ*) and of Fries medium (high ammonia,* ▲*) and incubated in Fries medium as in Fig. 2. The arrow indicates the beginning of stationary phase.*

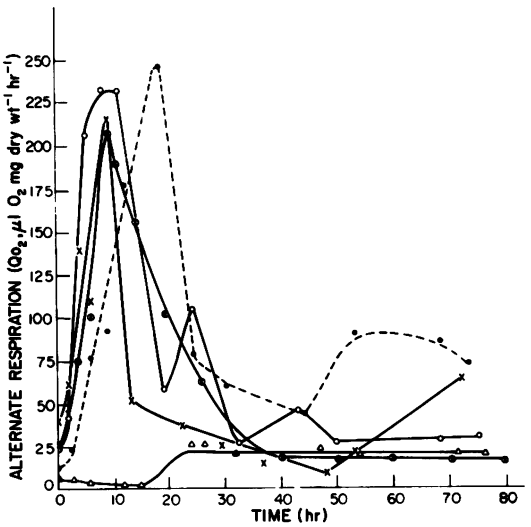

FIG. 7. *Specific alternate respiratory activities of mycelia of wild type and extrachromosomal mutants at various culture ages. Conditions and symbols as in Fig. 2. Alternate respiration is that fraction of total respiration which is cyanide resistant and sensitive to salicyl hydroxamate (see Materials and Methods).*

the alternate system. Other experiments (data not shown) indicate that cycloheximide inhibits the derepressed synthesis of the alternate system, so the increase in activity represents synthesis of component(s) of the alternate system and not an activation of a pre-existing system.

The rate and magnitude of derepression of the synthesis of the alternate system in wild type in the presence of chloramphenicol, as a function of number of mass doublings, were similar to the rate and magnitude in the mutants in the

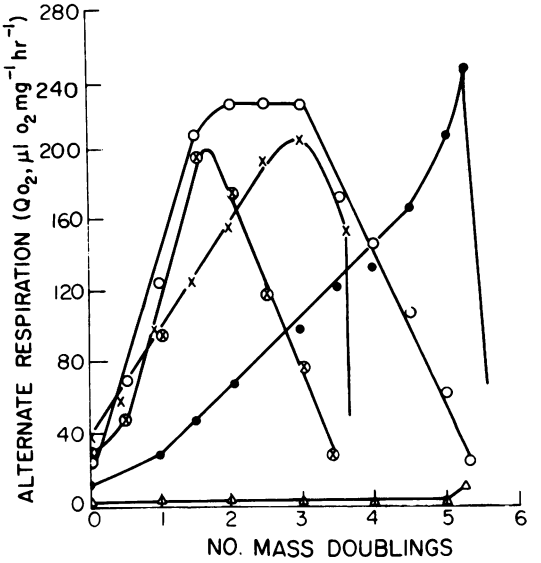

FIG. 8. *Specific alternate respiratory activities of mycelia of wild type and extrachromosomal mutants as a function of number of mass doublings. Conditions and symbols as in Fig. 2 and 7.*

absence of chloramphenicol. Therefore, the mutants appear to be fully derepressed in the synthesis of the alternate system.

In other experiments with the double mutant *arg-5* [*SG-1*], increases in both the terminal and alternate activities occurred in the presence, but not in the absence, of arginine supplement (B. L. Sauer, unpublished data). These results substantiate the conclusions derived from studies of the effects of inhibitors of protein synthesis on respiratory adaptation in wild type.

The composition of the culture medium (ammonium versus nitrate ion) used for conidial production had a marked effect upon respiratory activity of wild-type conidia (B. L. Sauer et al., in press) and the rate and magnitude of change of this activity during subsequent germination and growth (Fig. 9). The proportions of alternate and terminal respiratory activities were related in approximately a reciprocal manner (compare Fig. 6 and Fig. 9; Fig. 5A and B; Fig. 4 and Fig. 8; B. L. Sauer et al., in press).

DISCUSSION

Mitochondrial deoxyribonucleic acid (DNA) of *N. crassa* contains cistrons which encode proteins synthesized in mitochondria (5) as well as cistrons for ribosomal (25) and transfer ribonucleic acid (RNA; 5). The autonomy of mitochondria in their production of mitochondrial messenger RNA in another ascomycete, *Saccharomyces cerevisiae*, has recently been demonstrated (21).

The molecular bases and primary consequences of extrachromosomal mutations in *N. crassa* are not yet clearly understood. Alterations of the mitochondrial protein-synthesizing system, either in ribosomes (24) or transfer RNA (6), have been observed in the mutant [*mi-1*]. The proposal that the properties of mitochondrial structural protein from this mutant are altered (30) was retracted (32). Recent studies on the induction of the synthesis of mitochondrial DNA and RNA polymerase, methionyl-transfer RNA transformylase, cytochromes *b*, *c*, and c_1, and mitochondrial ribosomes led Barath and Küntzel to propose a dynamic model of the control of mitochondrial biogenesis in *N. crassa* (1). The results of the present studies indicate that the synthesis and catabolism of the mitochondrial respiratory chain may be subject to genetic and regulatory controls.

Genetic and regulatory mechanisms for the terminal respiratory system of N. crassa mitochondria. In wild type, synthesis of the terminal system was sustained at a rate equivalent to growth rate for at least four generations. In the fifth generation in the approach to stationary phase, synthesis stopped, and the specific activity of the system was diluted by one-half from the mass doubling. Later in stationary phase, catabolism or inactivation led to the loss of all activity. Conidia from stationary-phase cultures are also devoid of terminal activity (B. L. Sauer et al., in press). Pre-

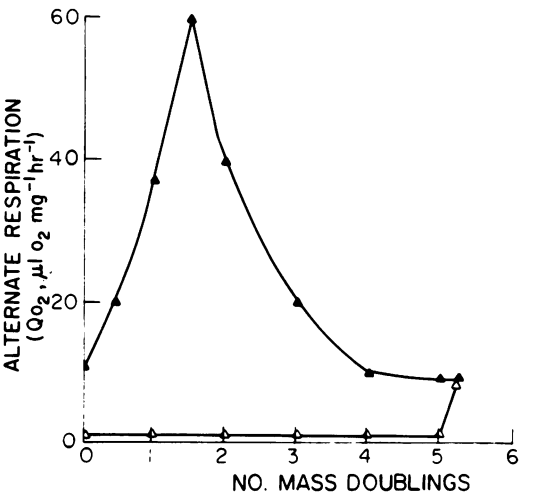

FIG. 9. *Influence of the preculture medium upon the change of alternate respiratory activity during growth of wild type. Conditions as in Fig. 6.*

mature cessation of the synthesis of the terminal system was caused by chloramphenicol, an inhibitor of protein synthesis in mitochondria. This premature cessation of synthesis then led to premature catabolism or inactivation of the system. Conversely, preculture conditions such as nitrogen source and concentration (co-repressor?) influence the subsequent rate and extent of increase of terminal activity without affecting the catabolic process (Fig. 6).

In the mutants, the capacity for the synthesis of the terminal system initially approached that of wild type, but net synthesis was not sustained as long as in wild type, and catabolism began at a relatively earlier time. The multiplicity of cycles of increase and decrease of terminal activity in some of the mutants, in contrast to wild type with only one cycle, also points to genetic alteration of the programs for synthesis and catabolism of the terminal system.

Other observations also suggest that catabolism of the terminal respiratory system may be involved in the regulation of its activity. Mitchell and Hertzenberg (22) observed an active "cytochromase" in [*mi-1*] and [*mi-3*] but not in wild type, and suggested that the destruction of the cytochromes of the terminal respiratory chain by the cytochromase might account for the observed deficiency in some of the cytochromes. Similarly, we have observed that at the onset of stationary phase the specific activity of a proteinase was three- to fivefold greater in [*mi-1*] than in wild type (Munkres, unpublished data). We have also observed that the molecular weight distribution of proteins synthesized by mitochondria in vivo in [*mi-1*] and [*mi-3*] is significantly different from that of wild type. Relative to wild type, the mutants exhibited a decrease in proportion of high-molecular-weight proteins and an increase in proportion of low-molecular-weight proteins (28; Neiss and Munkres, in preparation). Among several interpretations of these results, one is that proteolysis of these proteins is more active in the mutants.

Regulation of respiratory activities. In terms of the dynamic model of regulation of mitochondrial biogenesis in *N. crassa* proposed by Barath and Küntzel (1), a number of observations on the effects of mitochondrial mutations and inhibitors of mitochondrial protein synthesis may be explained. Barath and Küntzel (1) suggest that: (i) a mitochondrial-encoded protein may act as a repressor of the nuclear genes which encode for other mitochondrial proteins; and (ii) a nuclear-encoded protein may serve as repressor of mitochondrial genes.

Regulation of the synthesis of the proteins of the alternate and terminal respiratory systems of mitochondria may, therefore, be viewed as follows.

Conditions which lead to the absence or inactivation of the repressor from mitochondria may be: (i) inhibition of mitochondrial protein synthesis by chloramphenicol; (ii) mutation of a mitochondrial gene encoding the repressor; and (iii) high ammonium ion concentration in the medium (anti-repressor?) (B. L. Sauer et al., in press). Any of these three conditions would lead to a derepression of the synthesis of components of the alternate respiratory system and a repression of the synthesis of the components of the terminal system. Derepression inhibition of the protein synthesis of the alternate system by cycloheximide indicates that they are synthesized on cytoribosomes and probably encoded by nuclear genes.

Conditions ii and iii may not only affect the synthesis of some components of the terminal respiratory system, but also may lead to premature catabolism of the system. This program for catabolism normally may begin in stationary phase for wild type. By analogy, catabolism of a biosynthetic enzyme, tryptophan synthetase, in *N. crassa* may be caused by a protease which reaches maximal activity in stationary phase (31). Inhibition by chloramphenicol of mitochondrial protein synthesis in *Saccharomyces cerevisiae* also leads to the rapid loss of respiratory activity (9, 12). The loss of respiratory activity and respiratory enzyme activities in stationary-phase cultures of *S. cerevisiae* may be caused by lipid peroxidation damage (23). (Although phosphorylation was not measured in the present work, the view that any interruption of oxidative phosphorylation results in degradation of mitochondria in vivo was recently and extensively reviewed [20].)

A model in which nuclear genes encode for proteins of the alternate system and mitochondrial genes encode for some of the proteins of the terminal system, together with the mutual regulation of the two genomes by repressors according to Barath and Küntzel, could explain the basis for our observations that the specific activities of the two systems under various conditions are reciprocally related.

In the accompanying paper (7), the programs for ethanolic fermentation are compared with the programs for respiration.

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